

Functional alteration of PARL contributes to mitochondrial dysregulation in Parkinson's disease

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Molecular genetics has linked mitochondrial dysfunction to the pathogenesis of Parkinson's disease by the discovery of rare, inherited mutations in gene products that associate with the mitochondria. Mutations in PTEN-induced kinase-1 (*PINK1*), which encodes a mitochondrial kinase, and *PARKIN*, encoding an E3 ubiquitin ligase, are the most frequent causes of recessive Parkinson's disease. Recent functional studies have revealed that *PINK1* recruits *PARKIN* to mitochondria to initiate mitophagy, an important autophagic quality control mechanism that rids the cell of damaged mitochondria. *PINK1* is post-translationally processed into a cleaved form whose levels are tightly regulated, although the significance of this processing is unknown. Here we demonstrate that the mitochondrial protease presenilin-associated rhomboid-like (*PARL*) can affect the proteolytic processing of *PINK1* and that normal *PINK1* localization and stability requires *PARL*'s catalytic activity. *PARL* deficiency impairs *PARKIN* recruitment to mitochondria, suggesting *PINK1*'s processing and localization are important in determining its interaction with *PARKIN*. We sequenced the *PARL* gene in Parkinson's disease patients and discovered a novel missense mutation in a functional domain of *PARL*'s N-terminus. This *PARL* mutant is not sufficient to rescue *PARKIN* recruitment, suggesting that impaired mitophagy may be an underlying mechanism of disease pathogenesis in patients with *PARL* mutations.

INTRODUCTION

Parkinson's disease (PD) is the most common movement disorder in the world (1). It is a complex neurodegenerative disorder where known gene mutations account for <5% of all late-onset cases and ~50% of recessively inherited early-onset disease (2,3). Both biochemical and molecular genetic studies have highlighted mitochondrial dysregulation as a prominent mechanism in the pathogenesis of PD (4). Mutations in the genes for the mitochondrial-targeted protein PTEN-induced kinase-1 (*PINK1*) and the mitochondria-associated protein *PARKIN* are responsible for a large proportion of early-onset recessively inherited PD (5). *PINK1* has been characterized as being neuroprotective under conditions of

cellular stress and may play a role in regulating mitochondrial membrane dynamics (6,7). *PARKIN* is a primarily cytosolic E3 ubiquitin ligase that has been implicated in maintaining normal mitochondrial function and is recruited to damaged mitochondria (8,9). More recently, *PINK1* and *PARKIN* have been shown to play a role in the autophagic removal of damaged mitochondria, a process known as mitophagy. Several studies have established that *PINK1* functions to recruit cytoplasmic *PARKIN* to the mitochondria during depolarization, leading to the initiation of mitophagy (10–14). Using *Drosophila* as a model, we previously reported that the mitochondrial rhomboid protease (known as rhomboid-7 in flies) is a member of the *Pink1/Parkin* genetic pathway (15). Rhomboid-7 cleaves the initially mitochondrial-localized

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Pink1 as well as the mitochondrial protease Omi. This processing is proposed to regulate the localization and activity of Pink1 and Omi in the fly. Like *Drosophila* Pink1, mammalian PINK1 has been observed to undergo proteolytic processing (16,17). This led us to hypothesize that the mammalian ortholog of rhomboid-7, presenilin-associated rhomboid-like (PARL), might share the same biological activity within mitochondria. Indeed, PARL was recently shown to process the Omi protease, a reaction that required the co-factor Hax-1 (18). Herein, we show that PARL affects PINK1 processing and demonstrate that PINK1's subcellular localization and stability are regulated by PARL, which, in turn, influences PARKIN's recruitment to the mitochondria during induced mitophagy. Furthermore, we have identified a missense mutation in the N-terminal regulatory region of the PARL protease in PD patients and demonstrate that the resultant amino acid substitution affects PARL's biological activity.

RESULTS

PINK1 processing and stability are altered in PARL-deficient cells

Based on our observations in *Drosophila*, we hypothesized that mammalian PINK1 could be a substrate of PARL. PINK1 has a hydrophobic region in its N-terminus which could be the site of intramembrane proteolysis that leads to the two isoforms of PINK1 most often observed by western blotting (10,16,19,20). We first tested whether PINK1 expression was altered in mammalian cells lacking PARL. Due to the well-documented lack of effective PINK1 antibodies (21), transient transfections were performed with a C-terminal hemagglutinin (HA) epitope-tagged PINK1 in mouse embryonic fibroblasts (MEFs) derived from PARL^{-/-} mice and the parental ^{+/+} line (22). As reported by others (16), PINK1 was expressed as its predicted full-length form (~68 kDa, FL-PINK1) and a cleaved, processed form (~58 kDa, Δ1-PINK1), although these were both expressed at low levels in wild-type MEFs (Fig. 1A, lane 1). We also detected two smaller PINK1-specific bands (~54 and ~50 kDa, Δ2-PINK1 and Δ3-PINK1, respectively), which have been observed previously (23,24), but not characterized. Notably, when expressed in MEFs lacking PARL, we observed an increase in the ratio of FL-PINK1 to Δ1-PINK1 compared with control MEFs (Fig. 1A, lanes 2 and 4). Surprisingly, reintroducing wild-type PARL to PARL^{-/-} MEFs resulted in a reduction in all forms of PINK1, whereas the inactive catalytic mutant of PARL, p.Ser277Gly (25), did not affect PINK1 levels (Fig. 1A, lanes 3 and 4). Assessing PINK1 cleavage is confounded by the fact that Δ1-PINK1 is subjected to rapid constitutive proteosomal degradation (17,26,27). In order to follow the fate of Δ1-PINK1, we treated MEFs with the proteasome inhibitor MG132 (17,26,27). As reported previously in other cell types, MG132 treatment resulted in an accumulation of Δ1-PINK1 in wild-type MEFs (Fig. 1B, lane 2). In PARL^{-/-} MEFs treated with MG132, there was an accumulation of FL-PINK1 similar to that observed in untreated cells (Fig. 1B, lane 3). Strikingly, over expression of wild-type PARL in PARL^{-/-} MEFs resulted in the disappearance of

unprocessed FL-PINK1 and only Δ1-PINK1 could be detected (Fig. 1B, lane 4), suggesting that in untreated cells over-expressing PARL FL-PINK1 is processed completely into Δ1-PINK1 and subsequently degraded in a proteasome-dependent manner. Interestingly, a PINK1-specific band (~40 kDa, Δ4-PINK1) was detected in PARL^{-/-} MEFs that were not present in PARL^{+/+} cells (Fig. 1A, lanes 2 and 4, Fig. 1B, lanes 3 and 5). This observation, and the fact that, even in the absence of PARL, FL-PINK1 processing was not completely abolished, suggests that alternative PINK1 processing by another protease may be occurring in the absence of PARL. Taken together, these results show that PINK1 processing is altered in PARL-deficient cells. The ability of wild-type PARL but not catalytically inactive mutant PARL to restore PINK1 processing in PARL^{-/-} MEFs suggests that PINK1 may be a substrate of PARL. Rhomboid proteases are known to cleave their substrates within or near a hydrophobic transmembrane domain, resulting in the release of the substrate from the membrane (28,29), thus PINK1's N-terminal hydrophobic domain around amino acids 105–115 could be the site of PARL-dependent proteolysis. To investigate this further, we expressed an N-terminally truncated PINK1, Δ110-PINK1, and compared its size with that of the isoforms produced by wild-type PINK1. Δ110-PINK1 co-migrated very close to Δ1-PINK1 (Fig. 1C), suggesting that the proteolytic cleavage that converts FL-PINK1 to Δ1-PINK1 resides in or very close to the N-terminal hydrophobic domain.

PARL is required for normal PINK1 localization

PINK1 has been reported to localize to both the cytoplasm and the mitochondria. It is predominantly cytoplasmic under normal cellular conditions and can become primarily localized to the mitochondria during mitochondrial depolarization (Supplementary Material, Fig. S1A–C) (10,16,23,30,31). Because the subcellular localization of PINK1 is important with respect to mitochondrial function and mitochondrial quality control (10,30), we examined whether PARL had any influence on PINK1 localization within the cell. Using immunofluorescence, we observed that PINK1 is largely cytoplasmic with some mitochondrial staining in wild-type MEFs, which is consistent with reported localizations in other cell types (Fig. 2). Conversely, PINK1 was found almost exclusively associated with mitochondria in the absence of PARL (Fig. 2A and C). This differential localization of PINK1 was also demonstrated by biochemical fractionation of MEFs (Supplementary Material, Fig. S1D). Expressing wild-type PARL in PARL^{-/-} MEFs restored PINK1's predominant cytoplasmic localization, but the catalytic PARL mutant could not (Fig. 2B). We interpret these results to suggest that PARL is required for PINK1's release from the mitochondria after import. An alternative explanation is that PINK1's exclusively mitochondrial localization in PARL^{-/-} MEFs is the result of mitochondrial dysfunction caused by loss of PARL. However, extensive characterization of PARL^{-/-} cells has shown that mitochondrial respiratory function and membrane potential are not perturbed (22).

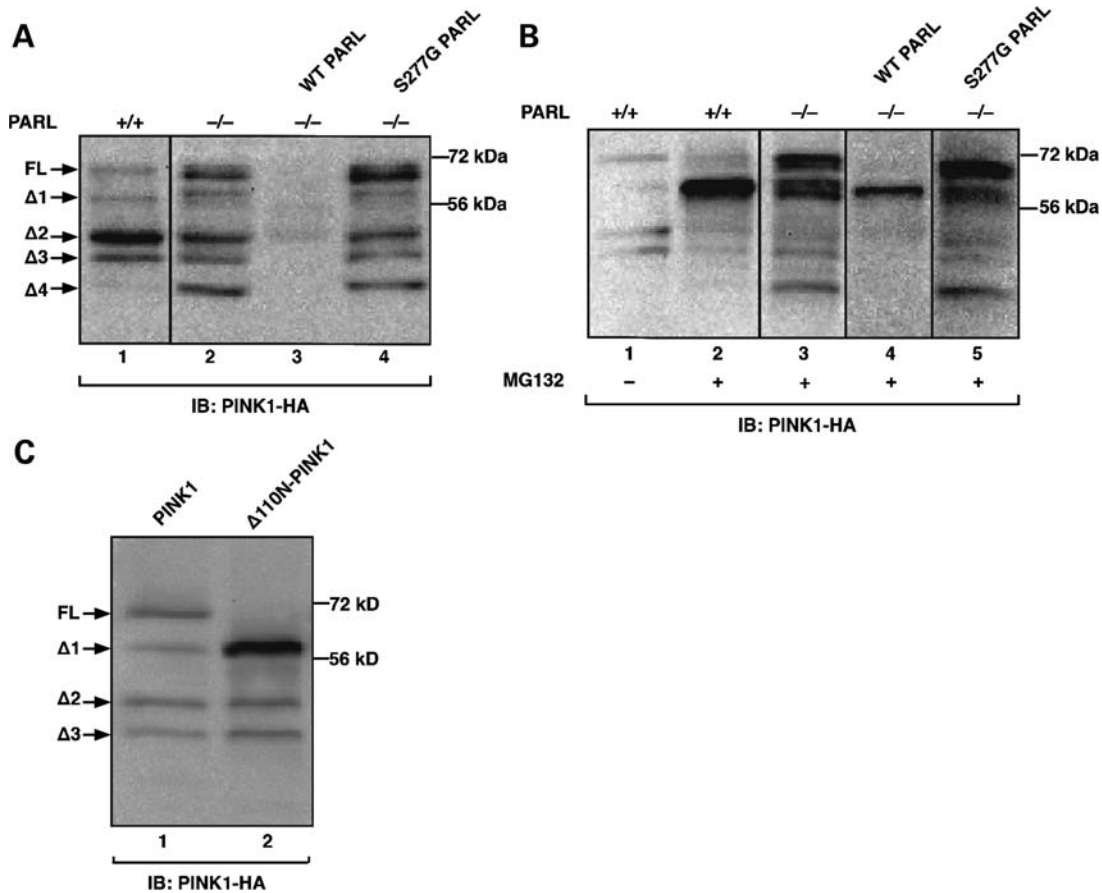


Figure 1. PINK1 processing is altered in PARL-deficient cells. (A) PINK1 processing is different in PARL^{+/+} and ^{-/-} MEFs. PINK1 was transiently transfected into PARL^{+/+} and ^{-/-} MEFs (lanes 1 and 2, respectively). PINK1 and wild-type (WT) PARL/S277G PARL catalytic mutant were co-transfected into PARL^{-/-} MEFs (lanes 3 and 4, respectively). Full-length PINK1 (~68 kDa, FL-PINK1), processed PINK1 (~58 kDa, Δ1-PINK1) as well as alternative PINK1 processing (~54 kDa, Δ2-PINK1 and ~50 kDa, Δ3-PINK1) were observed in the presence of PARL, whereas accumulation of full-length PINK1 as well as another alternative PINK1 processing (~40 kDa, Δ4-PINK1) were detected in the absence of PARL. Expression of WT PARL in PARL^{-/-} MEF resulted in reduction in all forms of PINK1; however, the catalytically inactive PARL mutant, S277G, did not affect PINK1 levels. Vertical lines between lanes indicate non-adjacent samples on the same gel/western blot. (B) PINK1 may be a substrate of PARL. PINK1 was transiently transfected into PARL^{+/+} and ^{-/-} MEFs (lanes 1, 2 and 3, respectively). PINK1 and WT PARL/S277G PARL catalytic mutant were co-transfected into PARL^{-/-} MEFs (lanes 3 and 4, respectively). Samples in lanes 2–5 were collected from cells that were treated with MG132 24 h prior to lysis. Compared with untreated samples, accumulation of Δ1-PINK1 was observed in the presence of PARL, whereas accumulation of FL-PINK1 was detected in the absence of PARL. Expression of WT PARL in PARL^{-/-} MEFs eliminated the accumulation of full-length PINK1; however, expression of the catalytically inactive PARL mutant, S277G, did not. Vertical lines between lanes indicate non-adjacent samples on the same western blot. (C) PARL-dependent cleavage site is near the PINK1 N-terminal hydrophobic domain. WT and N-terminally truncated PINK1 (Δ110-PINK1) were transiently transfected into HEK 293 cells. An intense band (~58 kDa) in lane 2 represents Δ110-PINK1. Δ1-PINK1 co-migrated very close to Δ110-PINK1.

A novel mutation in *PARL* is associated with PD

Given the interaction of PARL with PINK1, we further investigated the *PARL* gene as a functional candidate gene for PD. Our initial DNA sequencing of the *PARL* gene in a cohort of 230 PD patients (Table 1) revealed a novel mutation that was not present in 200 control samples. The mutation (NM_018622.5:c.230G>A) results in a substitution of a serine to an asparagine at codon 77 (p.Ser77Asn) in an individual with no family history of PD. Strikingly, this position is highly conserved (Fig. 3) and represents a critical site where auto catalytic processing is known to occur (32). To determine the frequency of this mutation within the PD population, we screened a further 2353 patients and 999 controls from various ethnic groups (Table 2) and identified a second patient with familial PD with the identical mutation. Neither

the c.230G>A nor any other variant affecting amino acid 77 in *PARL* was identified in the single nucleotide polymorphism database (dbSNP) or 1000 genomes.

Overall, the p.Ser77Asn variant was detected in the PD population at a frequency of 1 in 1291 (95% confidence range of 1 in 357 to 1 in 10 000).

S77N PARL mutants have altered N-terminal cleavage

Strikingly, the p.Ser77Asn substitution we detected in two PD patients affects the only known PARL regulatory site outside of its catalytic region. PARL undergoes a self-regulated proteolysis (β-cleavage) which may regulate its enzymatic activity (25,33). The cleavage is regulated by phosphorylation, and this requires the conserved serine at position 77. In

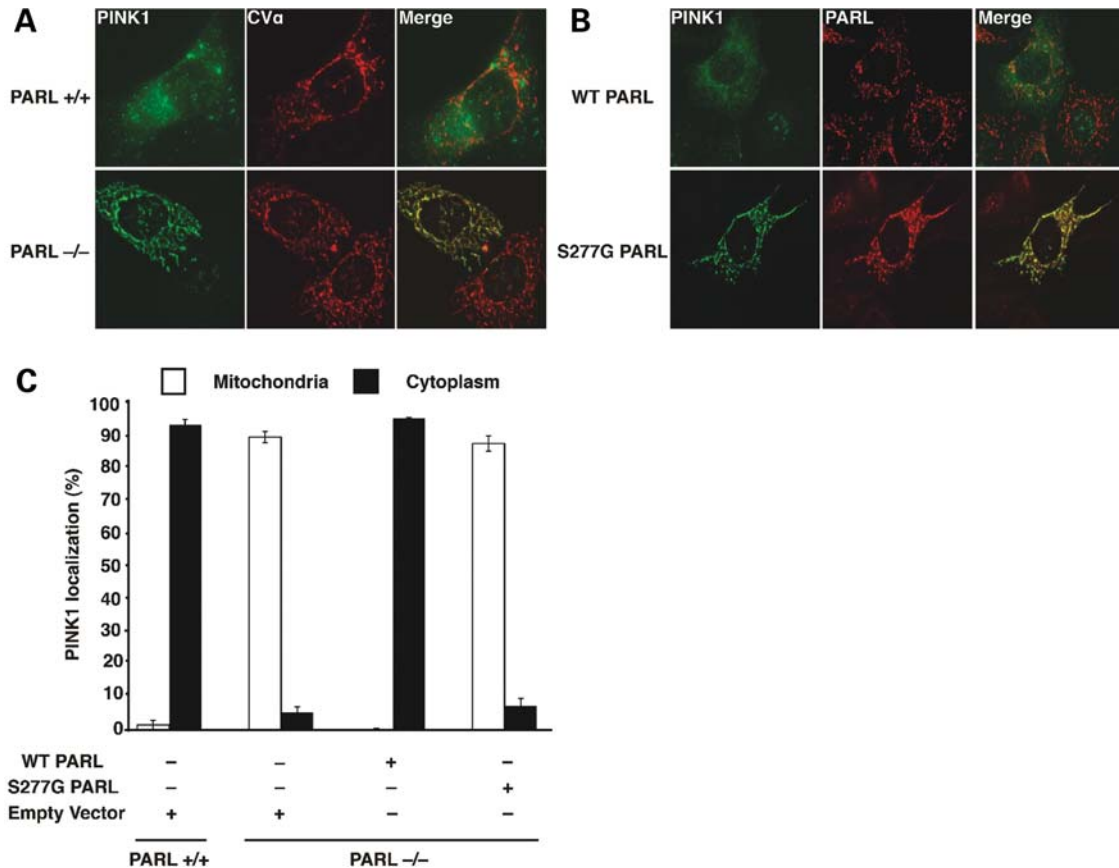


Figure 2. PARL activity is required for normal PINK1 intracellular localization. (A) PINK localization is regulated by the presence of PARL. MEFs derived from PARL^{+/+} (upper panels) and PARL^{-/-} mice (lower panels) were transiently transfected with PINK1. PINK1 localization (left panels, green) was compared with the mitochondrial protein CV- α (middle panels, red). The right panels show the merged images. PINK1 co-localized well with CV- α in PARL^{-/-} MEFs; however, the co-localization is weak in PARL^{+/+} MEFs. (B) PINK localization is regulated by the catalytic activity of PARL. MEFs derived from PARL^{-/-} mice were transiently co-transfected with PINK1 and WT PARL (upper panels) or the S277G PARL catalytic mutant (lower panels). PINK1 localization (left panels, green) was compared with PARL localization (a constitutively mitochondrial protein, middle panels, red). The right panels show the merged images. PINK1 relocalization from the mitochondria to the cytoplasm was achieved efficiently by WT PARL but not by the PARL catalytic mutant. (C) Quantification of the experiments as shown in (A) and (B).

Table 1. Patient characteristics of the initial PD cohort

PD cohort	230 individuals
Family history	
At least one affected relative	34%
At least one first degree affected relative	14%
Three or more affected relatives	2%
Age of onset < 50 years	27%
Average age of onset (range)	57.7 (31–92 years)
Ethnicity	
North or Central European	97%
French-Canadian	55%
Controls	
Average age at time of sample collection (range)	63.5 (24–86 years)
Anonymous Caucasian controls	200

addition, it has been reported that β -cleavage is required for PARL to induce mitochondrial fragmentation in an over-expression assay (25). To investigate the functional consequences of the p.Ser77Asn substitution, we expressed this mutant form of PARL in human embryonic kidney (HEK) 293T cells. As expected, p.Ser77Asn PARL does not

undergo β -cleavage (Fig. 4A). We next examined mutant PARL's ability to induce mitochondrial fragmentation. As reported previously, when wild-type PARL was over-expressed, it induced mitochondrial fragmentation, whereas p.Ser77Asn impaired the ability of PARL to induce mitochondrial fragmentation (Fig. 4B). This functional evidence supports the pathogenicity of p.Ser77Asn in PD.

PINK1-mediated PARKIN recruitment and light chain-3 conversion are impaired in PARL^{-/-} MEFs

PINK1 is required for the recruitment of PARKIN to damaged mitochondria to initiate mitophagy (10,30,31). Given the role of PARL in PINK1 processing and localization, we tested whether PARL is required for PARKIN's recruitment to mitochondria during carbonyl cyanide-m-chlorophenylhydrazone (CCCP)-induced mitophagy. As a control, PARKIN was efficiently recruited to mitochondria upon CCCP treatment in PARL^{+/+} MEFs (Fig. 5C, far right) and was found mostly in the cytoplasm when cells were not treated with CCCP (Fig. 5B). In contrast, PARKIN recruitment to mitochondria

<i>H. sapiens</i>	PD patients	58	RKVEPRRSDPGTSGEAYK	RSAL	IPPVEETVFYSPY	93
<i>H. sapiens</i>	NP_061092.3	58	RKVEPRRSDPGTSGEAYK	RSAL	IPPVEETVFYSPY	93
<i>P. troglodytes</i>	XP_001135897.1	58	RKVEPRRSDTGTSGEAYK	RSAL	IPPVEETVFYSPY	93
<i>C. lupus</i>	XP_545224.2	58	RKVEPRRSDTGTSGEAYK	RSAL	IPPVEETVFYSPY	93
<i>E. caballus</i>	XP_001496750.1	58	RKVEPRRSDTGTSGEAYK	RSAL	IPPVEETVFYSPY	93
<i>B. taurus</i>	NP_001015596.1	58	RKVEPRRSD--TSSEAYK	RSAL	IPPVEETAFYSPY	91
<i>M. musculus</i>	NP_001005767.1	56	RKVEPRRSDTGSSGEAYK	RSAL	IPPLEETVFYSPY	91
<i>R. norvegicus</i>	XP_001055224.1	56	RKVEPRRSDTGSSGEAYK	RSAL	IPPLEETVFYSPY	91
<i>D. melanogaster</i>	NP_523704.1	31	NSGHPAARSCRQIHSNRK	QSSN	LKP TTGEPAAAEQN	66

Figure 3. Amino acid sequence conservation observed in the region of the β -cleavage site of PARL. Orthologous amino acid sequences of PARL were aligned using CLUSTALW to demonstrate the high degree of sequence similarity in mammals. The position of the mutated amino acid is highlighted in red. Those amino acids required for β -cleavage are in the rectangle and the cleavage site is indicated by the arrow. The equivalent sequence of the *Drosophila* rhomboid-7 has been included in the alignment to indicate that the overall conservation of the PARL N-terminus is restricted to mammals.

Table 2. Patient characteristics for each series

Variable	PD cases	Controls
Taiwanese series (391 cases, 344 controls)		
Age	69 \pm 11 (31–89)	59 \pm 14 (22–101)
Gender (male)	221 (57%)	114 (33%)
Age at onset	59 \pm 12 (26–90)	NA
Canadian series (518 cases, 313 controls)		
Age	75 \pm 10 (23–95)	68 \pm 13 (22–95)
Gender (male)	325 (63%)	93 (30%)
Age at onset	62 \pm 11 (28–88)	NA
US series (313 cases, 348 controls)		
Age	73 \pm 11 (35–92)	72 \pm 11 (33–90)
Gender (male)	176 (56%)	190 (55%)
Age at onset	62 \pm 12 (16–85)	NA
Polish series (352 cases, 351 controls)		
Age	69 \pm 10 (38–92)	64 \pm 15 (23–97)
Gender (male)	222 (63%)	174 (50%)
Age at onset	57 \pm 12 (25–81)	NA
Irish series (327 cases, 355 controls)		
Age	66 \pm 10 (36–93)	71 \pm 22 (23–103)
Gender (male)	188 (57%)	127 (36%)
Age at onset	51 \pm 11 (18–77)	NA
Norwegian series (452 cases, 533 controls)		
Age	73 \pm 11 (45–99)	70 \pm 12 (43–106)
Gender (male)	274 (61%)	301 (56%)
Age at onset	59 \pm 11 (30–88)	NA

The sample mean \pm SD (minimum, maximum) is given for age and age at onset. Information regarding age at onset was unavailable for 8 patients in the Taiwanese series, 27 patients in the Canadian series, 10 patients in the US series, 7 patients in the Polish series and 103 patients in the Irish series.

upon CCCP treatment was significantly reduced in PARL $^{-/-}$ MEFs. Importantly, we were able to restore PARKIN recruitment by adding back wild-type PARL to the cells, but not the catalytic mutant (p.Ser277Gly; Fig. 5C). Interestingly, when the disease-linked p.Ser77Asn PARL mutant was introduced, PARKIN recruitment was not restored (Fig. 5C). To further substantiate a functional defect in mitophagy in the absence of PARL, we assayed the proteolytic conversion of the microtubule-associated protein-1 light chain-3 (LC3), a well-characterized marker for macroautophagy (34). Figure 5D shows that the CCCP-induced conversion of LC3-I to LC3-II is impaired in PARL $^{-/-}$ MEFs. Although LC3 conversion does not distinguish between mitophagy and general macroautophagy, these data are consistent with an impaired mitophagic response.

DISCUSSION

PINK1 plays an important role in maintaining mitochondrial health and protecting the mitochondria during cellular stress. One way in which PINK1 fulfills this function is by recruiting PARKIN during mitophagy, a process that allows the selective removal of damaged mitochondria. PINK1 has been localized to both the cytoplasm and the mitochondria and undergoes post-translational processing, producing at least three cleaved forms from the full-length protein. Little is understood about how PINK1 processing and localization affect the PINK1 function. Based on our previous studies in *Drosophila*, we hypothesized that the mitochondrial rhomboid protease, PARL, could be responsible for PINK1 processing and regulation. Using MEFs derived from PARL knockout mice, we show that PINK1 processing is indeed altered in the absence of PARL. We interpret our data to suggest that PARL is involved directly as the protease that cleaves PINK1. The accumulation of FL-PINK1 was observed in the absence of PARL and normal processing could be restored only by catalytically active exogenous PARL. Furthermore, a form of PINK1 truncated within its hydrophobic transmembrane domain, the proposed recognition site of rhomboid proteases, co-migrated with $\Delta 1$ -PINK1. Interestingly, a recent paper by Narendra *et al.* (10) ruled out PARL as the protease responsible for cleaving PINK using the same PARL $^{-/-}$ MEFs. This discrepancy may be explained by the fact that Narendra *et al.* did not stabilize $\Delta 1$ -PINK1 with MG132 in their analysis. Due to the alternative cleavage we observed in the absence of PARL, the effect on PINK1 processing is subtle without proteasome inhibition. Narendra *et al.* do see an increase in FL-PINK1 in PARL $^{-/-}$ MEFs, which is consistent with our observations in the absence of MG132. Our data do not completely exclude the possibility that PARL is affecting PINK1 by another mechanism, such as indirectly regulating PINK1 stability or import pathways into the mitochondria. However, our conclusion that PARL is the protease that cleaves PINK is most consistent with the known function of rhomboid proteases as well as our previous observations in *Drosophila*.

The fact that PINK1 undergoes a number of post-translational cleavage events, even in the absence of PARL, requires further investigation. Our data suggest that PINK1 is subject to alternative processing by a different protease in the absence of PARL. This alternative processing produces alternative forms of PINK1 but also leads to the production

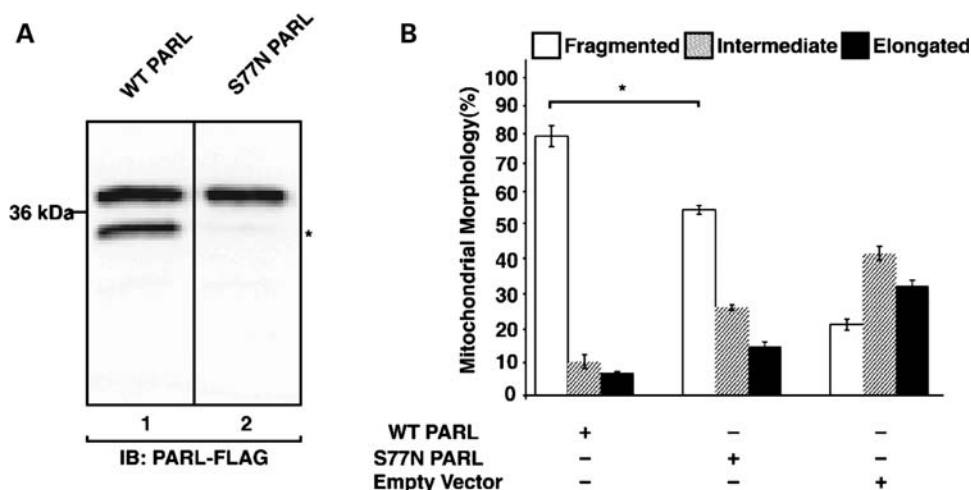


Figure 4. The PD-linked PARL mutation affects its function. (A) The PD-linked PARL mutation S77N abrogates the N-terminal β -cleavage of PARL. Both full-length and cleaved PARL were detected in cells that express WT PARL, but the well-characterized N-terminal PARL cleavage product was abolished in cells that express the S77N PARL mutant. *Cleaved form of PARL. (B) The PD-linked PARL mutation S77N alters the ability of PARL to fragment mitochondria. WT PARL, S277G and S77N PARL mutants were transiently transfected into COS-7 cells and mitochondrial morphologies of transfected cells were analyzed by immunofluorescence microscopy by the mitochondrial protein CV- α . Mitochondrial morphology was grouped into fragmented, intermediate and elongated. * $P < 0.005$.

of $\Delta 4$ -PINK1. Intriguingly, over expression of PARL results in a reduction of all forms of PINK1, except $\Delta 1$ -PINK1 when stabilized by MG132. In this case, the complete conversion of FL-PINK1 to $\Delta 1$ -PINK1 may deplete the pool of unprocessed PINK1 available for alternative cleavage. As there are no other characterized intramembrane proteases in the mitochondrion, we would predict that the alternative cleavage site does not reside within PINK1's transmembrane domain. Indeed, the alternatively processed PINK1 appeared to migrate slightly higher than $\Delta 1$ -PINK1 in PARL^{-/-} MEFs than in wild-type MEFs (Fig. 1A, lanes 1 and 2), suggesting that this cleavage may be occurring just N-terminal to the transmembrane domain. To further characterize PINK1 processing, the precise site of proteolysis for each of the various PINK1 cleavage products needs to be determined.

PINK1 has a mitochondrial targeting sequence but is predominantly observed in the cytoplasm. This may be explained by our observation that PINK1 becomes restricted to the mitochondria in the absence of PARL. In this scenario, PINK1 is imported into the mitochondria where its transmembrane domain anchors it to the inner mitochondrial membrane (IMM) and it cannot escape. Upon PARL cleavage, PINK1 is released from the membrane and is subsequently able to access the cytoplasm. This model is supported by the fact that others have observed $\Delta 1$ -PINK1 in the cytoplasm by biochemical fractionation (19). Furthermore, $\Delta 1$ -PINK1 is selectively degraded by the proteasome, a process that is limited to the cytoplasm (27). Thus, PARL appears to play a role in controlling PINK1's access to the cytoplasm. If PINK1 requires PARL for release from the IMM, it would also explain why we see impaired PARKIN recruitment in PARL^{-/-} MEFs during induced mitophagy, which is characterized by PINK1 accumulation on the outer mitochondrial membrane (OMM). Interestingly, it has been suggested that PINK1's neuroprotective function is not restricted to the mitochondria (23). The significance of the cytoplasmic pool of PINK1 and whether

or not there are cytoplasmic targets of PINK1 phosphorylation warrant further study.

Our results are seemingly at odds with a report that PINK1 is localized in the OMM with its kinase domain facing the cytoplasm (21), whereas PARL is a well established resident of the IMM (25). However, several other studies have suggested that PINK1 does in fact reside within the IMM (35–37). Our data do not exclude a model in which PINK1's sub mitochondrial residence is dependent on other factors, such as cell type or conditions of depolarization. Indeed, full-length PINK1 has been shown to accumulate in the OMM during CCCP-induced mitochondrial depolarization (10). Determining the sub mitochondrial localization of each of the PINK1 cleavage products under normal and depolarizing conditions will be necessary to resolve the apparent discrepancies in reported PINK1 localization.

Mitochondrial dysregulation has emerged as a key mechanism in the pathogenesis of PD. Uncovering a role for PARL in regulating PINK1 led us to investigate the PARL gene as a functional candidate for PD. Although no known linkage, or overt association with the PARL locus at 3q27.1 has been identified, this would not be surprising if the disease allele frequency were very low. While ~1300 controls were screened, the frequency of the variant, on its own, is low enough that we cannot reject the hypothesis that this is a very rare variant with a true frequency of 2/3782 (~0.05%), without sequencing very large numbers of people. Barring functional verification, the challenge of validating rare disease-causing variants is daunting, potentially requiring the screening of 70 000 individuals (38). However, the lack of such a variant in dbSNP and other databases such as 1000 genomes, and the high degree of conservation, presumably due to the invariant restrictions required for β -cleavage of PARL, all support a pathogenic nature to this variant (32). Furthermore, functional mapping of the PARL β -cleavage demonstrated its sensitivity to amino acid substitutions where the *in vitro* mutants PARL

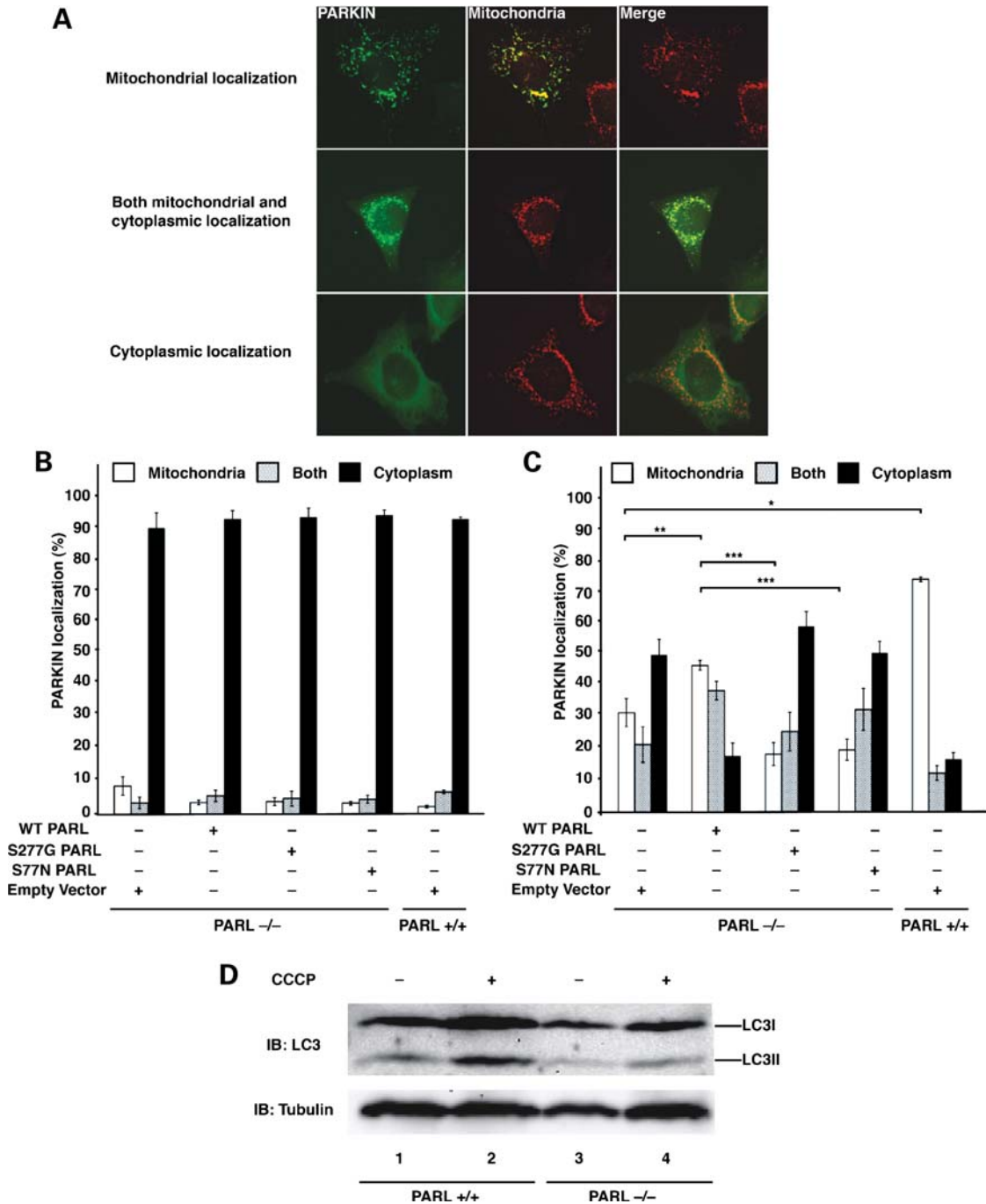


Figure 5. PARL is required for efficient PARKIN-dependent mitophagy. (A) Immunofluorescence images demonstrating the differential categorization of PARKIN localization in the experiments quantified in (B) and (C). Top panels show mitochondrial-localized PARKIN, middle panels show PARKIN localization to both mitochondria and cytoplasm and bottom panels show cytoplasmic only localization of PARKIN. (B) PARKIN is found only in the cytoplasm when not treated with CCCP, regardless of the experimental conditions, as described in (C). (C) Efficient mitochondrial recruitment of PARKIN to damaged mitochondria requires PARL and is impaired in the PD-linked PARL mutation. PARKIN and WT, catalytic mutant or PD-linked mutant PARL (WT PARL, S277G PARL and S77N PARL) were transiently transfected into PARL^{+/+} and ^{-/-} PINK1 MEF lines that express PINK1. Localization of PARKIN was compared with the mitochondrial marker CV- α by immunofluorescence microscopy and categorized into mitochondria localized, cytoplasmic localized or both. * $P < 0.001$; ** $P < 0.005$; *** $P < 0.03$. (D) LC3 conversion is impaired in PARL ^{-/-} MEFs. Analysis of total cell lysates by western blotting of PARL^{+/+} and ^{-/-} MEFs upon CCCP-induced mitophagy demonstrates a reduction in LC3-II production from LC3-I.

p.Arg76Glu, p.Ser77Glu, p.Ala78Glu and p.Leu79Glu were all shown not to be subjected to β -cleavage (32). Given that there are no insertions or deletions in the P β domain during the 100 million years of mammalian evolution [Fig. 3 (39)],

the P β domain must have been subjected to strong selection pressures, probably due to the functional constraints of the mitochondrial rhomboid protease within higher vertebrates (32). Finally, the demonstration of a functional and pathogenic

outcome of the p.Ser77Asn substitution, which disrupts PARL β -cleavage affecting PARL activity in a mitochondrial fragmentation assay, provides further evidence that it is a bona fide pathogenic mutation. Furthermore, p.Ser77Asn PARL was not able to restore PARKIN mitochondrial recruitment in PARL $^{-/-}$ MEFs during induced mitophagy.

In sum, our data expand the function of PARL in maintaining proper mitochondrial function to possibly include a role in regulating mitophagy. Further study is required to determine the role PARL-mediated PINK1 processing plays in PARKIN recruitment during mitophagy. Most importantly, our data suggest that the mutation p.Ser77Asn, observed only in patients with PD, can impair PARL's function and implicate PARL dysregulation in PD pathogenesis. Given the unique position of this mutation with respect to phosphorylation-dependent cleavage, further studies should focus on the identification of the kinases and phosphatases that regulate PARL β -cleavage. The identification and functional characterization of PARL p.Ser77Asn provides us with a useful tool and the opportunity to better understand the role of PARL in the pathogenesis of PD.

MATERIALS AND METHODS

Antibodies

The following antibodies were used: rabbit anti-HA (Sigma), mouse M2 anti-FLAG (Sigma), mouse anti-CV α (Mitosciences), rabbit and mouse horseradish peroxidase-conjugated secondary antibodies (Jackson Labs), rabbit and mouse Alexa Fluor secondary antibodies (Invitrogen).

DNA expression clones

Human PINK1 cDNA was TOPO[®] cloned into pENTR[™] and subsequently C-terminally tagged with a triple HA epitope using the Gateway[®] recombination system (Invitrogen). Human pSG PARL (gift of B. De Strooper) was mutagenized using the QuikChange[®] (Stratagene) method to make S277G and S77N PARL and confirmed by sequencing. N-terminally GFP-tagged PARKIN was a gift of D. Park.

Cell culture and transfection

PARL $+/+$ and $-/-$ MEFs (a gift of B. De Strooper) (22), COS-7 and HEK 293T cells were transfected using FuGENE 6 (Roche). Ten micromolar MG132 treatment for 24 h was used to inhibit the proteasome. Twenty micromolar CCCP treatment for 24 h was used to induce mitochondrial depolarization.

Genetic screening of *PARL* in PD patients

For the initial evaluation of the *PARL* gene, exons were amplified by PCR using oligonucleotide primers flanking each exon (primer sequences available on request). The PCR products were treated with ExoSAP-IT[™] (USB, Cleveland, OH, USA) and were sequenced in both directions with individual PCR primers and the BigDye[™] v3.1 terminator cycle

sequencing kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3130xl.

Subsequent genotyping of the PARL variant p.Ser77Asn was performed on a Sequenom MassArray iPLEX platform (San Diego, CA, USA) (primer sequences are available on request) and analyzed with Typer 4.0 software.

Immunoblotting

PINK1 expression in MEFs was detected by immunoprecipitation of HA-tagged PINK1 from cell lysates using rabbit HA followed by immunoblotting with mouse HA. Cell lysis and immunoprecipitation with Protein-G Sepharose (Roche) were performed according to the manufacturer's instructions. Samples were electrophoresed on 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes, blocked in 5% skim milk powder in Tris-buffered saline overnight and subsequently incubated with primary and secondary antibodies. Chemiluminescence detection was performed on a Versdoc imager (Bio-Rad).

Immunofluorescence and quantification of mitochondrial morphology

MEFs were seeded onto glass cover slips and subsequently transfected. To prepare samples for immunofluorescence analysis, cells were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde (PFA), washed again with PBS, permeabilized in 0.1% Triton X-100 and then blocked overnight in goat serum before incubation with primary and secondary antibodies in 10% goat serum. Cells were imaged using a Zeiss AxioVision fluorescence microscope. Localization of PINK1 and PARKIN was quantified by scoring the subcellular localization of the tagged protein in 100 cells in three separate experiments. *P*-values were calculated using the Student's *t*-test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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